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- (54) Title: SELF-CONTAINING LACTOCOCCUS STRAIN

(57) Abstract: The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds.

SELF-CONTAINING *Lactococcus* STRAIN**Field of the invention**

The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds. Moreover, said *Lactococcus* can be transformed with prophylactic and/or therapeutic molecules and can, as such, be used to treat diseases such as inflammatory bowel diseases.

Background of the invention

Lactic acid bacteria have long time been used in a wide variety of industrial fermentation processes. They have generally-regarded-as-safe status, making them potentially useful organisms for the production of commercially important proteins. Indeed, several heterologous proteins, such as Interleukin-2, have been successfully produced in *Lactococcus* spp (Steidler *et al.*, 1995). It is, however, unwanted that such genetically modified micro organisms are surviving and spreading in the environment.

To avoid unintentional release of genetically modified microorganisms, special guidelines for safe handling and technical requirements for physical containment are used. Although this may be useful in industrial fermentations, the physical containment is generally not considered as sufficient, and additional biological containment measures are taken to reduce the possibility of survival of the genetically modified microorganism in the environment. Biological containment is extremely important in cases where physical containment is difficult or even not applicable. This is, amongst others, the case in applications where genetically modified microorganisms are used as live vaccines or as vehicle for delivery of therapeutic compounds. Such applications have been described e.g. in WO 97/14806, which discloses the delivery of biologically active peptides, such as cytokines, to a subject, by recombinant non-invasive or non-pathogenic bacteria. WO 96/11277 describes the delivery of therapeutic compounds to an animal – including humans - by administration of a recombinant bacterium, encoding the therapeutic protein. Steidler *et al.* (2000) describe the treatment of colitis by administration of a recombinant *Lactococcus lactis*, secreting interleukin-10. Such a

usage of a self-containing and transform d *Lactococcus* to deliver prophylactic and/or therapeutic molecules in order to prevent and/or treat diseases.

Brief description of the figures

5 **Figure 1:** Map of the MG1363 *thyA* locus

Figure 2: Schematic representation of the different expression modules as present on pOThy plasmids ands genomic integrants of hIL-10. Black parts represent original *L. lactis* MG1363 genetic information, white parts represent recombinant genetic information.

10 **Figure 3:** PCR identification of Thy11 (Thy11 1.1 and Thy11 7.1 represent individually obtained, identical clones). Standard PCR reactions were performed by using aliquots of saturated cultures of the indicated strains as a source of DNA template. Panel A shows an agarose gel of the products of the indicated PCR reactions. Panel B shows the positions at which primers attach in the *thyA* (1), upstream (2) or downstream (3)

15 PCR's. Oligonucleotide primers used: (1): ATgACTTACgCAgATCAA~~Ag~~TTTTT and TTAAATTgCTAAATCAAATTCAATTg (2): TCTgATTgAgTACCTTgACC and gCAATCATAATTggTTTTATTg (3): CTTACATgACTATgAAAATCCg and cTTTTTTATTATTAgggAAAGCA.

20 **Figure 4:** PCR identification of Thy11, Thy12, Thy15 and Thy16. Standard PCR reactions were performed by using three days old colonies of the indicated strains as a source of DNA template.

Panel A shows the positions at which primers attach in the upstream (1), downstream (2) or *thyA* (3), PCR's. Oligonucleotide primers used: (1): ATgACTTACgCAgATCAA~~Ag~~TTTTT and TTAAATTgCTAAATCAAATTCAATTg (2): TCTgATTgAgTACCTTgACC and gCAATCATAATTggTTTTATTg (3): CTTACATgACTATgAAAATCCg and cTTTTTTATTATTAgggAAAGCA

25 Panel B shows an agarose gel of the products of the indicated PCR reactions.

Figure 5: Southern blot analysis of the indicated strains. Chromosomal DNA was extracted and digested with the indicated restriction enzymes. Following agarose gel electrophoresis the DNA was transferred to a membrane and the chromosome structure around the *thyA* locus was revealed by use of DIG labelled *thyA* or hIL-10 DNA fragments (panel A). Panel B shows a schematic overview of the predicted structure of the *thyA* locus in both MG1363 and Thy11.

diluted in TFM or TFM supplemented with 50µg/ml of thymidine (T50). CFU counts were determined at different time points: t=0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 20 hours. This shows that Thy12 viability is severely impaired in the absence of thymidine.

Figure 12: Intestinal passage and viability: *L. lactis* MG1363 was transformed with the plasmid pLET2N which carries a chloramphenicol (Cm) resistance marker. *L. lactis* Thy12 was transformed with the plasmid pT1NX which carries an erythromycin (Em) resistance marker. Of both strains 10⁹ bacteria were resuspended in BM9 (6 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l NH₄Cl, 0,5 g/l NaCl in 25 mM NaHCO₃ + 25 mM Na₂CO₃), mixed and inoculated in three mice at t=0h. Faeces were collected of the time intervals -1 to 0, 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 9, 9 to 10 and 10 to overnight. All samples were resuspended in isotonic buffer and appropriate dilutions were plated on GM17 (M17 medium, Difco, St.Louis supplemented with 0,5% glucose) plates containing either Cm, Em or Em+ 50µg/ml thymidine. Colony forming units for the different plates are represented in the graph.

15

Description of the invention

It is the objective of the present invention to provide a suitable biological containment system for *Lactococcus*.

A first aspect of the invention is an isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene. Preferably, said defective thymidylate synthase gene is inactivated by gene disruption. Even more preferably, said *Lactococcus* sp. is *Lactococcus lactis*. A special embodiment is a *Lactococcus* sp. strain, preferably *Lactococcus lactis*, more preferably a *Lactococcus lactis* MG1363 derivative, whereby the thymidylate synthase gene has been disrupted and replaced by an interleukin-10 expression unit. Said interleukin-10 expression unit is preferably, but not limited to, a human interleukin-10 expression unit or gene encoding for human interleukin-10.

Another aspect of the invention is the use of a strain according to the invention as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene. Still another aspect of the invention is a transformed strain of *Lactococcus* sp. according to the invention, comprising a plasmid that does not comprise an intact thymidylate synthase gene. Another aspect of the invention relates to a transformed strain of *Lactococcus* sp. comprising a gene or expression unit encoding a prophylactic and/or therapeutic molecule such as interleukin-10. Consequently, the present invention also relates to the usage of a transformed strain

invention further demonstrates that the transformed strains surprisingly pass the gut at the same speed as the control strains and shows that their loss of viability is indeed not different from that of the control strains. However, once said strain is secreted in the environment, e.g. in the faeces, it is not able to survive any longer.

- 5 The transforming plasmid can be any plasmid, as long as it cannot complement the *thyA* mutation. It may be a selfreplicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, the integrative plasmid itself may be used to create the mutation, by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated.
- 10 Preferably, the active *thyA* gene is replaced by double homologous recombination by a cassette comprising the gene or genes of interest, flanked by targeting sequences that target the insertion to the *thyA* target site. It is of extreme importance that these sequences are sufficiently long and sufficiently homologous to obtain to integrate the sequence into the target site. Preferably, said targeting sequences consist of at least
- 15 100 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 100 contiguous nucleotides of SEQ ID N°2 at the other side; more preferably, said targeting sequences consists of at least 500 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 500 contiguous nucleotides of the SEQ ID N° 2 at the other side; most preferably, said targeting sequences consists of
- 20 SEQ ID N°1 at one side of the gene of interest and SEQ ID N°2 at the other side, or said targeting sequences consist of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest, preferably
- 25 said targeting sequences consist of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest, most preferably said targeting sequences consist of at least 1000 nucleotides that are at
- 30 least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest . The percentage identity is measured with BLAST, according to Altschul *et al.* (1997). A preferred example of a sequence, homologous to SEQ ID N°1 is given in

locus as determined in the present invention are given by SEQ ID N° 19, 20, 21, 22 respectively.

The *thyA* replacement is performed by making suitable replacements in a plasmid borne version of the *thyA* target, as described below. The carrier plasmid is a

5 derivative of pORI19 (Law *et al.*, 1995) a replication defective plasmid, which only transfers the erythromycin resistance to a given strain when a first homologous recombination, at either the 5' 1000bp or at the 3'1000bp of the *thyA* target. A second homologous recombination at the 3' 1000bp or at the 5' 1000bp of the *thyA* target yields the desired strain.

10 The *thyA* gene is replaced by a synthetic gene encoding a protein which has the *L. lactis* Usp45 secretion leader (van Asseldonk *et al.*, 1990) fused to a protein of identical amino acid sequence than: (a) the mature part of human-interleukin 10 (hIL-10) or (b) the mature part of hIL-10 in which proline at position 2 had been replaced with alanine or (c) the mature part of hIL-10 in which the first two amino acids had 15 been deleted; (a), (b) and (c) are called hIL-10 analogs, the fusion products are called Usp45-hIL-10.

15 The *thyA* gene is replaced by an expression unit comprising the lactococcal P1 promotor (Waterfield *et al.*, 1995), the *E. coli* bacteriophageT7 expression signals: putative RNA stabilising sequence and modified gene10 ribosomal binding site (Wells and Schofield, 1996).

20 At the 5' end the insertion is performed in such way that the ATG of *thyA* is fused to the P1-T7Usp45-hIL-10 expression unit.

5' agataggaaaattcatgacttacgcagatcaagtttt...thyA wild type
gattaagtcatcttacctctt...P1-T7-usp45-hIL10

25 5' agataggaaaattcatggattaagtcatcttacctctt...thyA⁻, P1-T7-usp45-hIL10

Alternatively, at the 5' end the insertion is performed in such way that the *thyA* ATG is not included:

30 5' agataggaaaattcacttacgcagatcaagtttt...thyA wild type
gattaagtcatcttacctctt...P1-T7-usp45-hIL10

5' agataggaaaattcgattcatcttacctctt...thyA⁻, P1-T7-usp45-hIL10

integrative plasmid pT1HIL10apxa. Figure 8 (panel A and B) further demonstrates that all mutants produce a significant amount of h-IL 10.

Figure 9 shows the production of hIL-10 by the *L. lactis* strains LL108 carrying either pOThy11, pOThy12, or pOThy16. Quantification (by ELISA) of hIL-10 present in the

- 5 culture supernatant of the indicated strains. The N-terminal protein sequence of the recombinant hIL-10 was determined by Edman degradation and was shown identical to the structure as predicted for the mature, recombinant hIL-10. The protein showed full biological activity. LL108 is a *L. lactis* strain carrying a genomic integration of the repA gene, required for replication of pORI19 derived plasmids such as pOThy11,
10 pOThy12, pOThy15 or pOThy16. This strain was kindly donated by dr. Jan Kok, University of Groningen. The plasmids pOThy11, pOThy12, pOThy15 and pOThy16 carry the synthetic human IL-10 gene in different promotor configurations (see Fig. 2), flanked by approximately 1kB of genomic DNA derived from the thyA locus, upstream and downstream from thyA. These plasmids were used for the construction of the
15 genomic integration as described.

The effect of the thymidilate synthase deletion on the growth in thymidine less and thymidine supplemented media was tested; the results are summarized in figures 10 and 11. Absence of thymidine in the medium strongly limits the growth of the mutant,

- 20 and even results in a decrease of colony forming units after four hours of cultivation. Addition of thymidine to the medium results in an identical growth curve and amount of colony forming units, compared to the wild type strain, indicating that the mutant doesn't affect the growth or viability in thymidine supplemented medium. Fig. 11 clearly demonstrates that Thy12 viability is severely impaired in the absence of thymidine.

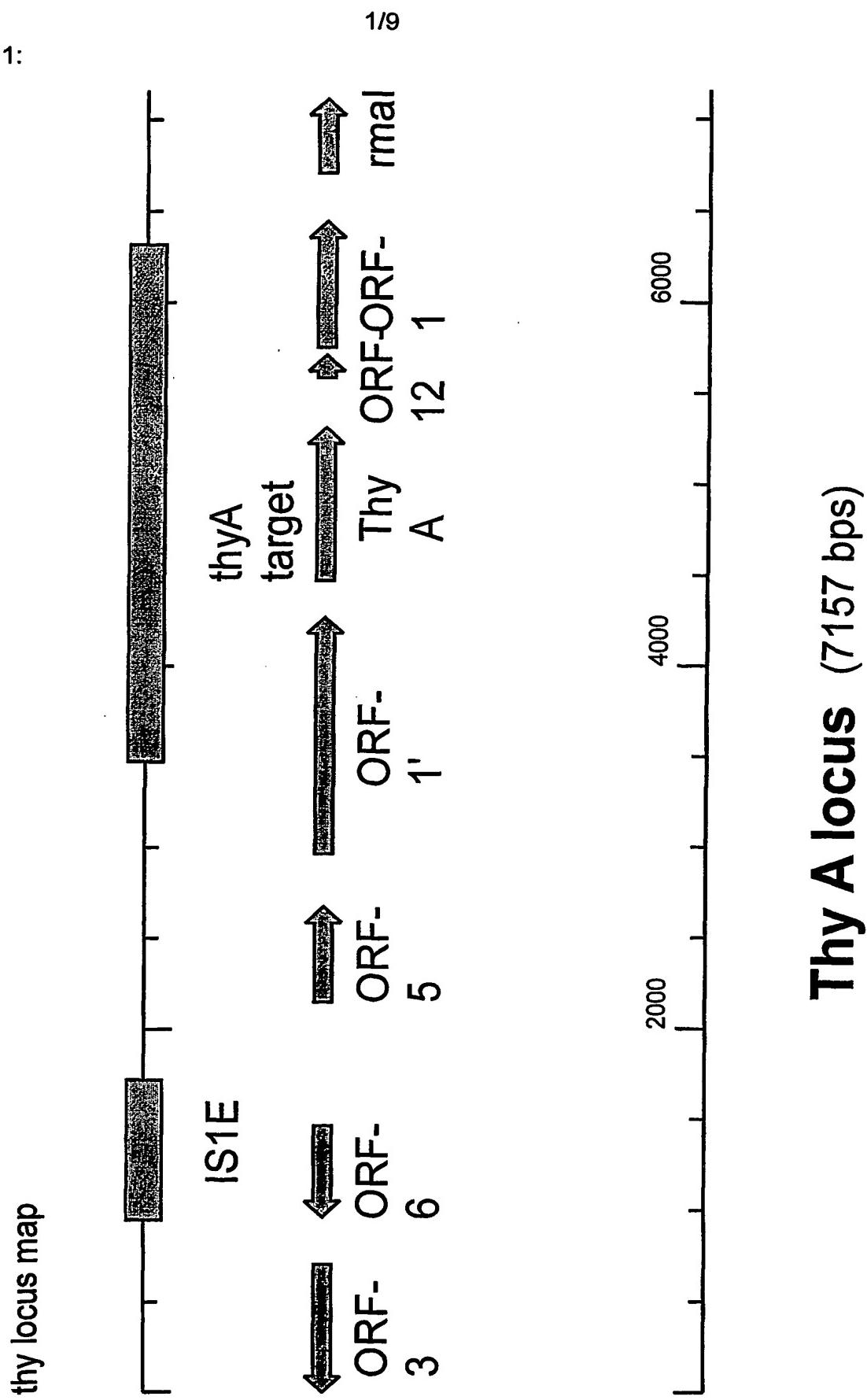
- 25 Fig. 12 finally shows that *L. lactis* Thy12 passes the intestine of the mice at the same speed as MG1363. Loss of viability does not appear different between Thy12 and MG1363. Thy12 appear fully dependent on thymidine for growth, indicating that no Thy12 bacteria had taken up a foreign thyA gene.

Claims

1. An isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene.
2. An isolated strain of *Lactococcus* sp. according to claim 1, whereby said gene is inactivated by gene disruption.
- 5 3. An isolated strain of *Lactococcus* sp. according to claim 1 or 2, whereby said *Lactococcus* sp. is *Lactococcus lactis*.
4. The use of a strain of *Lactococcus* sp. according to any of the claims 1-3 as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene.
- 10 5. A transformed strain of *Lactococcus* sp. according to any of the claims 1-3, comprising a transforming plasmid that does not comprise an intact thymidylate synthase gene.
6. A transformed strain of *Lactococcus* sp. according to any of the claims 1-4 comprising a gene encoding a prophylactic and/or therapeutic molecule.
- 15 7. A transformed strain of *Lactococcus* sp. according to claim 6 wherein said prophylactic and/or therapeutic molecule is interleukin-10.
8. The use of a transformed strain of *Lactococcus* sp. according to any of the claims 5-7 for the delivery of prophylactic and/or therapeutic molecules.
- 20 9. A pharmaceutical composition comprising a transformed strain of *Lactococcus* sp. according to any of the claims 5-7.
10. The use of a transformed strain of *Lactococcus* sp. according to any of the claims 6-7 for the preparation of a medicament to treat inflammatory bowel diseases.

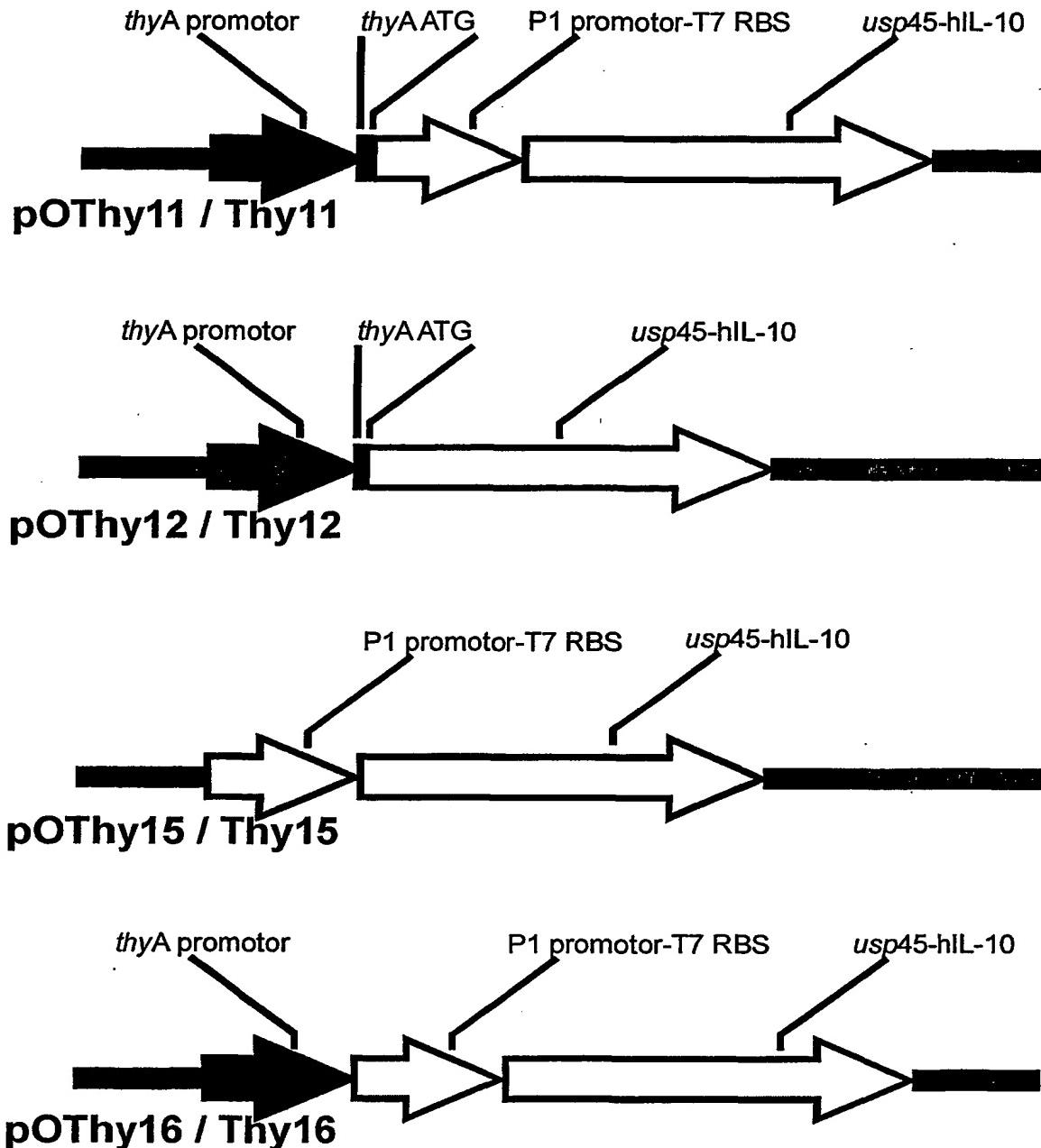
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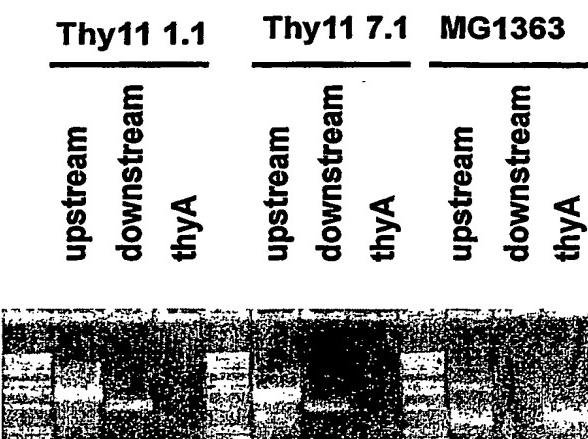
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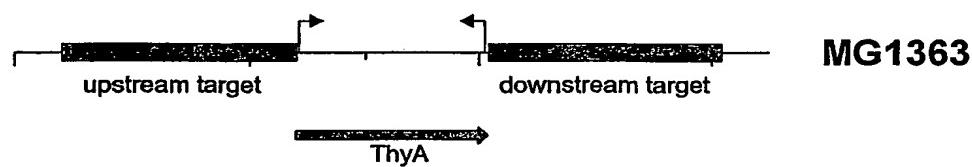
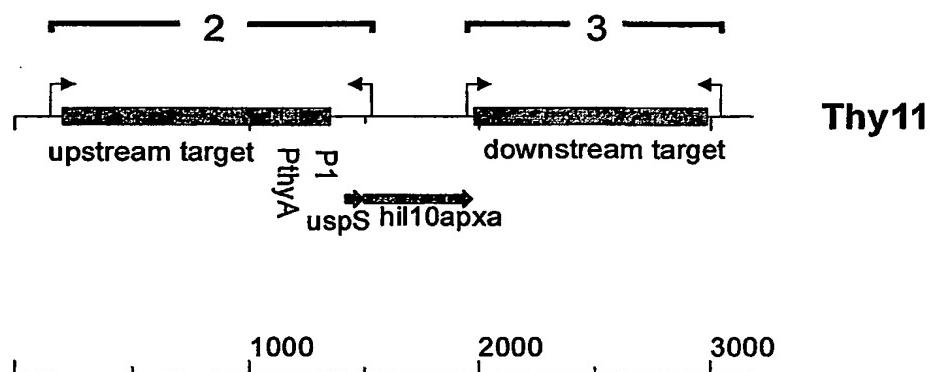


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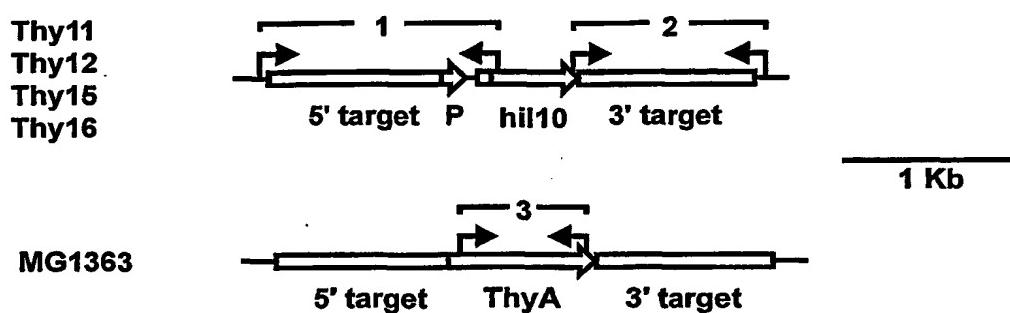
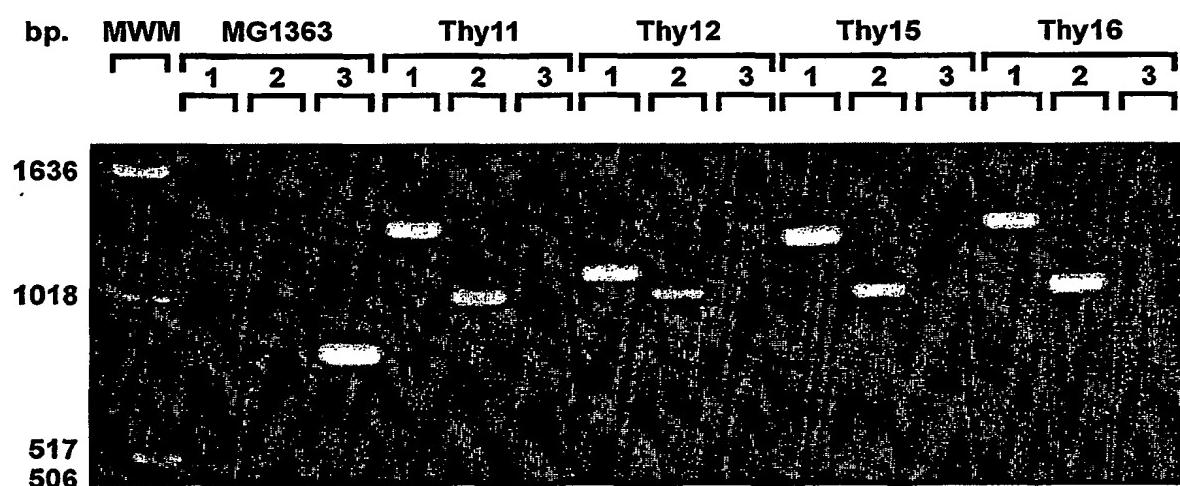
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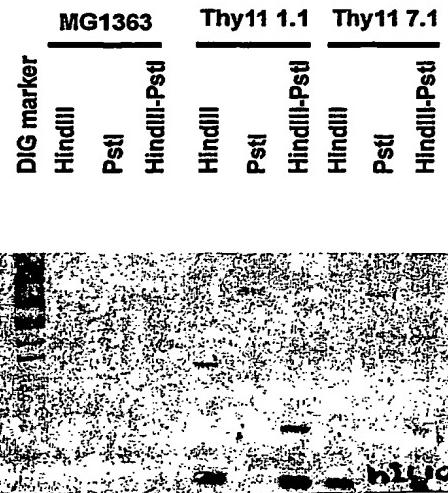
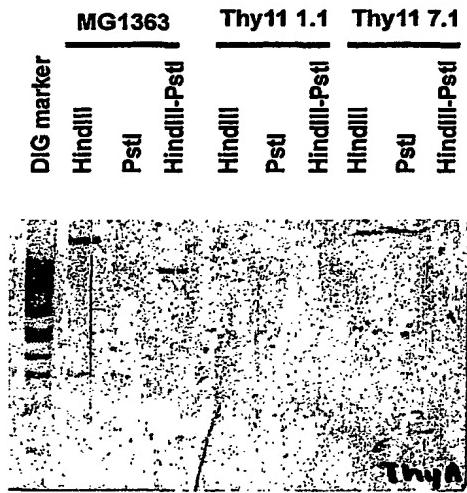
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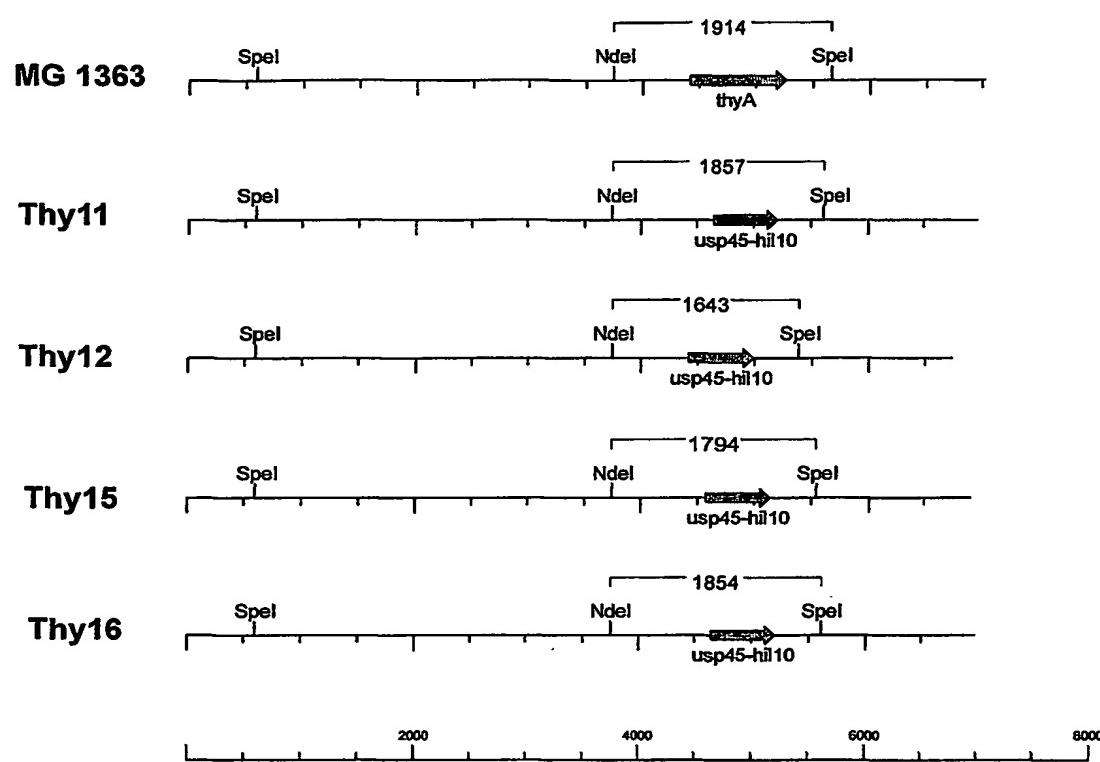
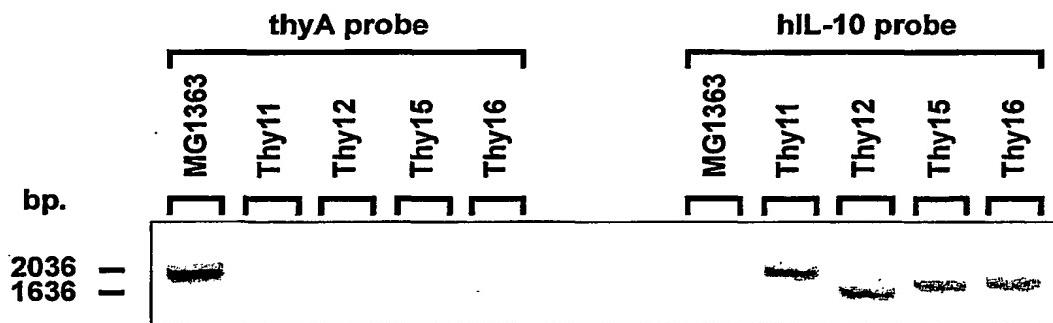
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Figure 5:

A

6/9

Figure 6:

A**B**

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Figure 7:

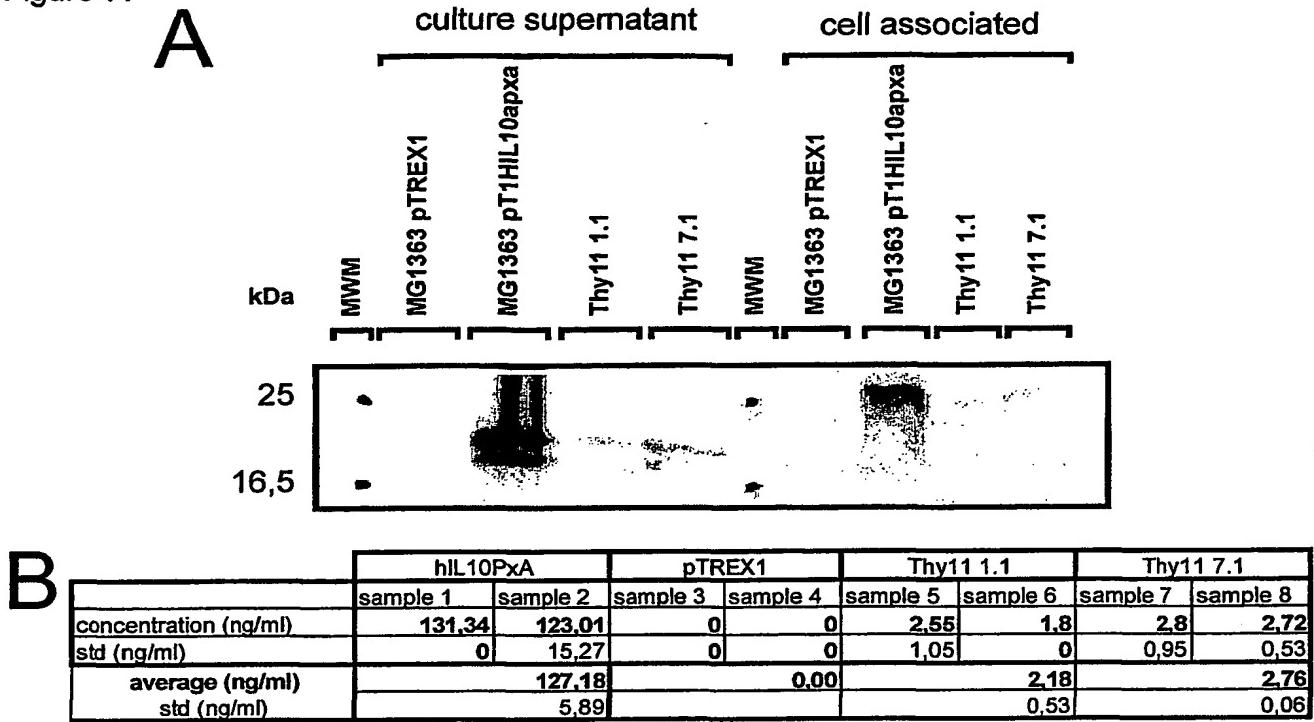
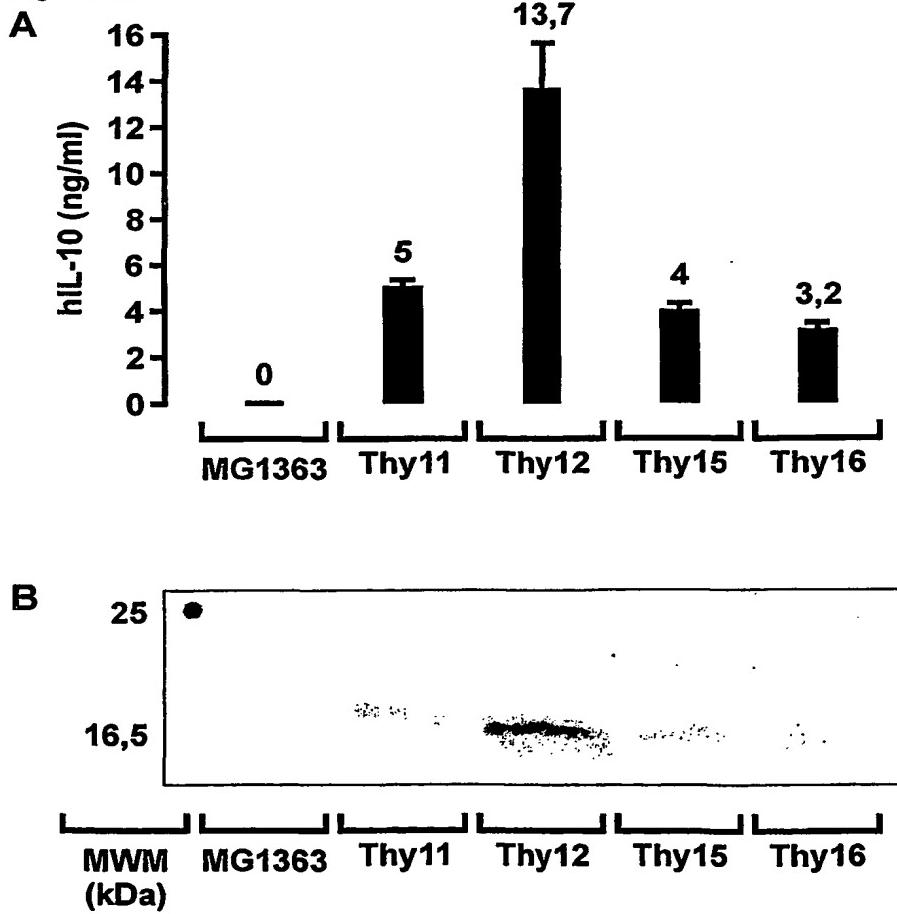


Figure 8:



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Figure 9:

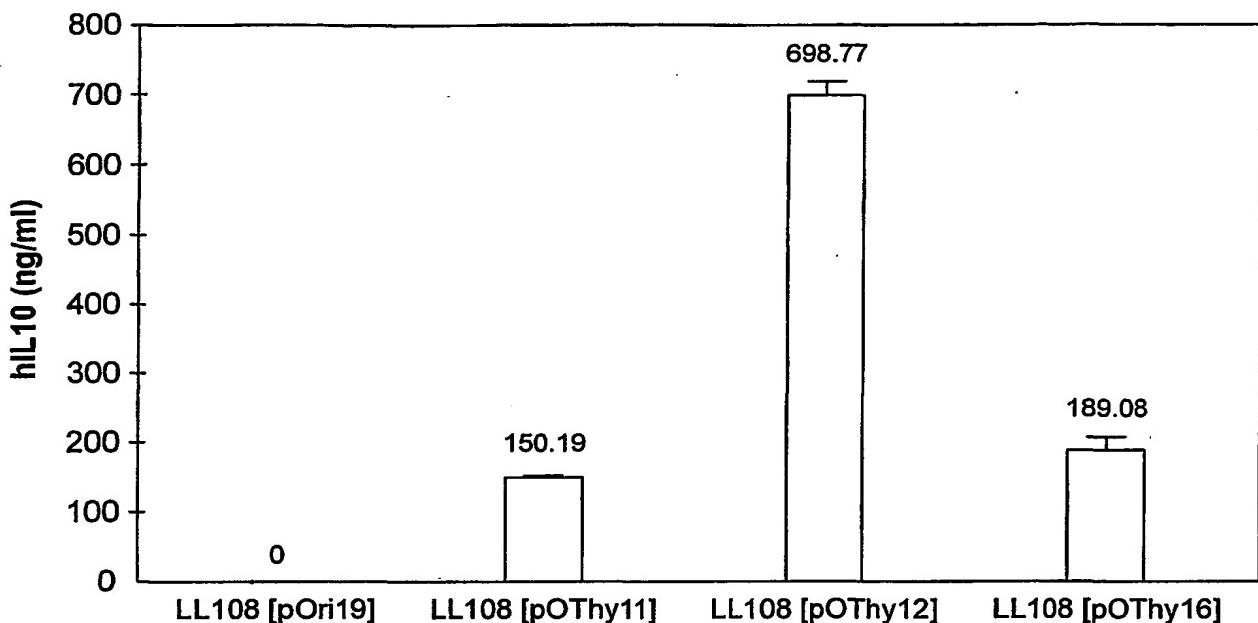
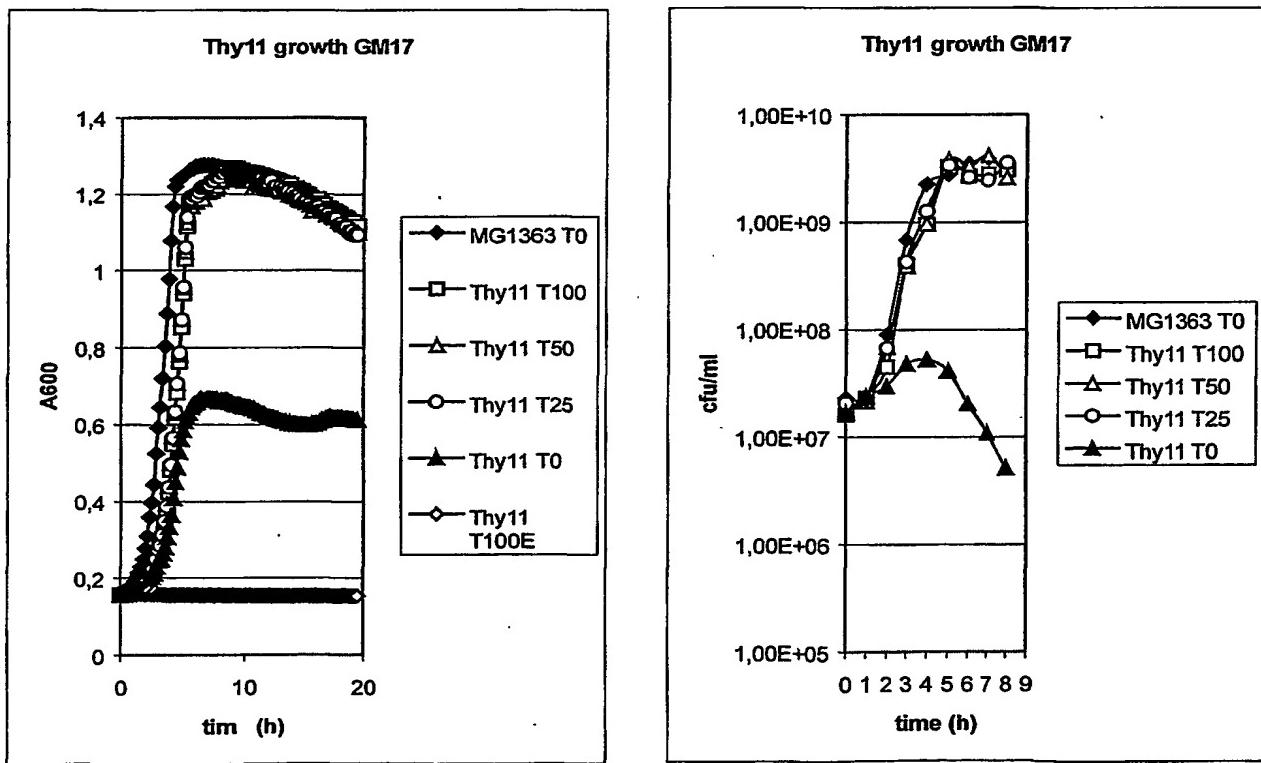


Figure 10:



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Figure 11:

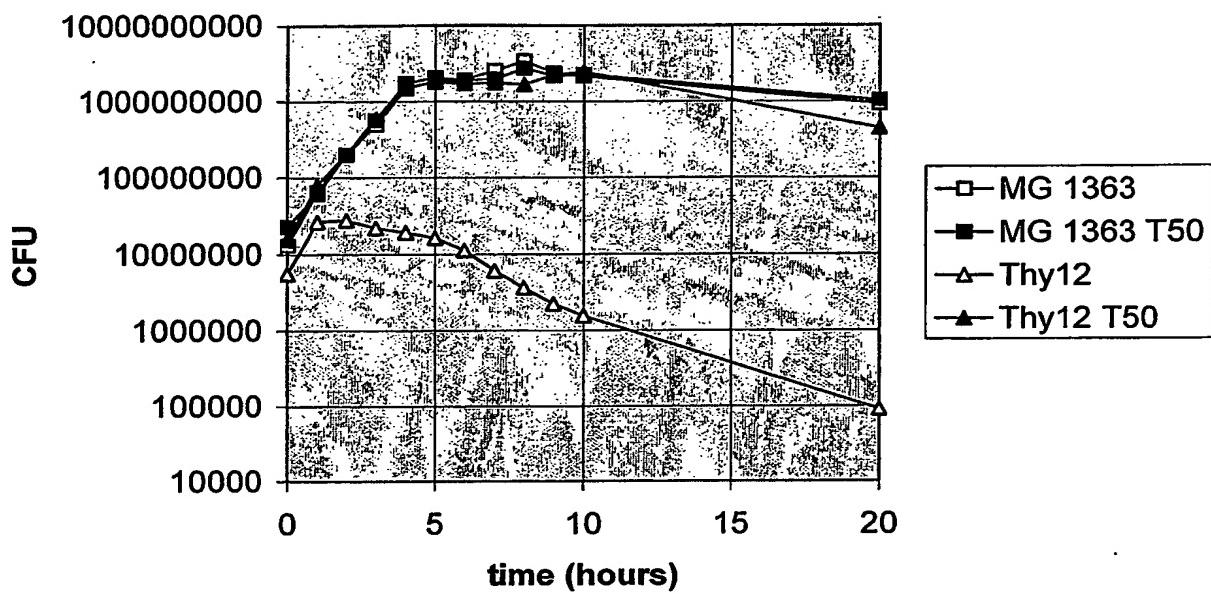
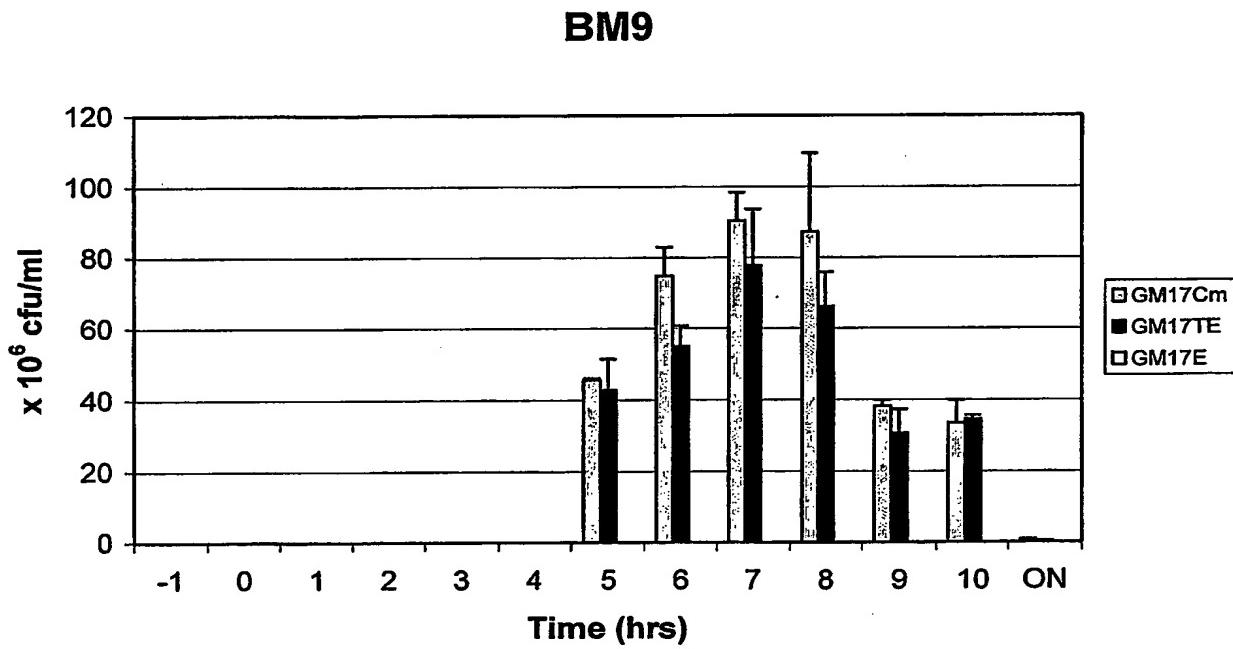


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V085.ST25.txt

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Asn Ala Arg Pro Lys	Tyr Lys Asp Gly Gln Met	Ala Asn Ser Lys Tyr				
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Val Thr Gly Ser Phe	Val Thr Tyr Asp Leu Gln Lys Gly	Glu Phe Pro				
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Ile Thr Thr Leu Arg	Pro Ile Pro Ile Lys Ser Ala Ile Lys Glu Leu					
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Met Trp Ile Tyr Gln	Asp Gln Thr Ser Glu Leu Ser Val	Leu Glu Glu				
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Lys Tyr Gly Val Lys	Tyr Trp Gly Glu Trp Gly Ile Gly Asp Gly Thr					
90	95	100				
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V085.ST25.txt

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V085.ST25.txt

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Pro Arg Leu Val Leu Asn Val Pro Asp Gly Thr Asn Phe Phe Asp Ile
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V085.ST25.txt

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45 50 55	
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75 80 85	
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V085.ST25.txt

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35 40 45

Leu Gln Lys Gly Glu Phe Pro Ile Thr Thr Leu Arg Pro Ile Pro Ile
50 55 60

Lys Ser Ala Ile Lys Glu Leu Met Trp Ile Tyr Gln Asp Gln Thr Ser
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Glu Leu Ser Val Leu Glu Glu Lys Tyr Gly Val Lys Tyr Trp Gly Glu
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V085.ST25.txt

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V085.ST25.txt

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